Penicillin formation by cell-free extracts of *Streptomyces clavuligerus*. Behaviour of aminoadipyl-modified analogs of the natural peptide precursor δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV)

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Six analogs of ACV, the biosynthetic precursor of the penicillin nucleus, have been synthesized, in which the δ-(L-α-aminoadipyl) moiety has been replaced by : β-(L-aspartyl), γ-(L-glutamyl), δ-(D-α-aminoadipyl), adipyl, glycyl-δ-(L-α-aminoadipyl), and *N*-acetyl-δ-(L-α-aminoadipyl). The penicillins having the adipyl, glycyl-δ-(L-α-aminoadipyl), and *N*-acetyl-δ-(L-α-aminoadipyl) side chains have also been synthesized. Several improvements have been made in earlier routes to ACV and related peptides, including a simplified preparation of a dipeptide precursor, and a new synthesis of α-aminoadipic acid from lysine. A new reagent, 2-acetoximino-2-phenylacetonitrile, has been synthesized, which allows ready *N*-acetylation of amino hydroxyacids in aqueous acetone at room temperature. The peptide analogs have been examined as substrates of the enzyme isopenicillin N synthetase, which converts ACV to isopenicillin N in the presence of oxygen, ferrous ions, and ascorbate. The enzyme has been isolated from the prokaryotic organism *Streptomyces clavuligerus*, and has been employed either as a crude salt precipitate or in semi-purified form, freed from other enzymes of the penicillin–cephalosporin pathway. With the crude enzyme preparation, three of the analogs are active substrates, viz., adipyl, glycyl-δ-(L-α-aminoadipyl), and *N*-acetyl-δ-(L-α-aminoadipyl), but the latter two are converted, only via ACV, to isopenicillin N, the normal cyclization product. That the crude enzyme preparation contains a protease that deacylates *N*-substituted ACV analogs to ACV is confirmed, *inter alia*, by the behaviour of the purified enzyme; of the various analogs, only the adipyl compound is an active substrate, but the conversion of this analog to carboxybutylpenicillin proceeds with only 1–2% efficiency, compared to the natural substrate.

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On a synthétisé six analogues de l'ACV, le précurseur biosynthétique du noyau de la pénicilline, dans lesquel on a remplacé l'unité δ-(L-α-aminoadipyl) par les unités suivantes: β-(L-aspartyl), γ-(L-glutamyl), δ-(D-α-aminoadipyl), adipyl, glycyl-δ-(L-α-aminoadipyl) et *N*-acétyl-δ-(L-α-aminoadipyl). On a également synthétisé des pénicillines ayant des groupes adipyl, glycyl-δ-(L-α-aminoadipyl) et *N*-acétyl-δ-(L-α-aminoadipyl) comme chaînes latérales. On a apporté plusieurs améliorations aux voies d'accès à l'ACV et aux peptides apparentés que nous avions décrites antérieurement; ces améliorations comprennent entre autre une préparation simplifiée d'un dipeptide précurseur ainsi qu'une nouvelle synthèse de l'acide α-aminoadipique à partir de la lysine. On a synthétisé un nouveau réactif, l'acétoxymino-2 phényl-2 acétonitrile, qui permet d'effectuer facilement une *N*-acétylation des hydroxy-acides aminés en opérant dans l'acétone aqueuse et à la température ambiante. On a étudié le comportement des analogues peptidiques comme substrats de l'enzyme isopénicilline *N*-synthétase qui transforme l'ACV en isopénicilline N en présence d'oxygène, d'ions ferreux et d'ions ascorbate. On a isolé l'enzyme à partir de l'organisme prokaryotique *Streptomyces clavuligerus* et on l'a utilisé soit sous la forme d'un sel précipité brut ou sous une forme semi-purifiée, alors que l'enzyme a été débarassé des autres enzymes de la voie pénicilline–céphalosporine. Avec la préparation brute de l'enzyme, les trois analogues portant les groupes adipyl, glycyl-δ-(L-α-aminoadipyl) et *N*-acétyl-δ-(L-α-aminoadipyl) sont des substrats actifs; toutefois, les deux derniers composés ne sont transformés en isopénicilline N, le produit normal de la cyclisation, que via l'ACV. Le fait que la préparation brute de l'enzyme contient un protéase qui peut enlever les groupes acétyles de l'ACV pour les transformer en ACV est confirmé, entre autre, par le comportement de l'enzyme purifié; des divers analogues, seul le composé portant un groupe adipyl s'avère un substrat actif, mais l'efficacité de la transformation de cet analogue en carboxybutylpénicilline n'est que de 1 à 2% par rapport au substrat naturel.

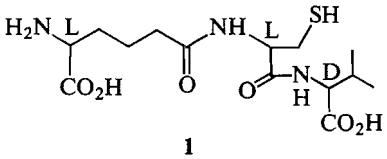
[Traduit par le journal]

Introduction

Streptomyces clavuligerus is a Gram positive, filamentous bacterium which produces a variety of β-lactam compounds including penicillin N, cephamycin C, and clavulanic acid (1, 2). The biosynthetic versatility of this organism, in terms of the synthesis of β-lactam compounds, has prompted an investigation of the enzymatic steps involved in the natural production of these antibiotics (3–5). Concurrent studies on the penicillin and cephalosporin-producing enzymes of *Cephalosporium acremonium* have been undertaken by several groups, and continue at the present time (6–8). One of these enzymes, variously referred to as cyclizing enzyme (3), cyclase (9), or isopenicillin N synthetase (10), leads to oxidative cyclization of the linear tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (**1**) (ACV) into isopenicillin N (**2**). This enzymatic step constitutes the single β-lactam ring forming reaction which leads from the amino acid oxidation level to penicillins and cephalosporins (cephamycins).

In both the prokaryotic (*S. clavuligerus*) and eukaryotic (*C. acremonium*) systems, this oxidative cyclization is carried out by an enzyme with remarkably similar characteristics. In both cases, the enzymes are soluble, require iron and ascorbate as cofactors, and consume molecular oxygen (3, 6, 9, 11).

As noted above, *S. clavuligerus* also produces clavulanic



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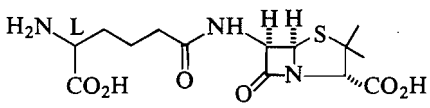

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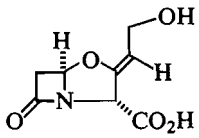
acid (**3**), a non-classical β-lactam compound which contains oxygen in the nucleus. The nature of the β-lactam ring forming enzyme associated with clavulanic acid biosynthesis is not known. It is conceivable that a single cyclizing enzyme of broad specificity could lead to the synthesis of penicillin–cephalosporin type compounds and also clavulanic acid (12). It seems more likely, however, that clavulanic acid biosynthesis involves a different pathway (13). Investigations into the substrate specificity of the cyclizing enzyme of *C. acremonium* have already indicated this enzyme to have a narrow substrate specificity (6, 9, 14).



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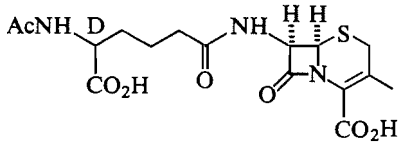
The present work is concerned with the behaviour of the cyclizing enzyme of *S. clavuligerus* towards analogs of ACV modified in the aminoadipyl residue. Certain specific modifications were of particular interest, because some β-lactam producing organisms also synthesize peptides, penicillins, and cephalosporins with side chains other than aminoadipyl. For example, ACV was first isolated from the mycelium of *Penicillium chrysogenum* (15), but was later found (16) to be contaminated with an additional peptide containing glycine. The structure and stereochemistry of ACV were established by Loder and Abraham (17) who isolated the peptide from the mycelium of *Cephalosporium* sp. C91, together with a tetrapeptide containing glycine, in addition to aminoadipic acid, cysteine, and valine. The constitution Aad-Cys-Val-Gly was suggested for this tetrapeptide, but a shortage of material prevented proof of this sequence. A peptide, apparently identical to Loder and Abraham's tetrapeptide, has been isolated from the β-lactam producing organism *Paecilomyces persicinus* P10 (18), and found to have the constitution Gly-Aad-Cys-Val (GACV). It was suggested that ACV is a precursor of penicillins, and that GACV may be a precursor of cephalosporins.

However, it has since become clear (3–5) that ACV is a precursor of both penicillins and cephalosporins. The biosynthetic significance of GACV therefore remains unknown.

N-Acetyldesacetoxycephalosporin C (**4**) has been isolated from a mutant of *C. acremonium* which is blocked in the synthesis of cephalosporin C (19). More recently, *N*-acetylisopenicillin N has been isolated from the culture broth of *Streptomyces tokunonensis* sp. nov., an organism that also produces isopenicillin N, penicillin N (**5**), and several carbapenems (20). The compound is considered in this case to be a shunt metabolite of isopenicillin N.

Cephalosporins containing fewer than six carbons in the side



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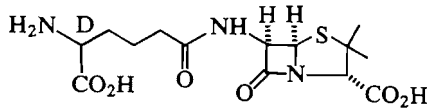

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chain, or lacking an amino group in the side chain, have been isolated from *Cephalosporium* spp. (21) and *Streptomyces* spp. (22). The side chains of these compounds are thought to be derived from the aminoadipyl side chains of cephalosporin precursors.

In view of these various observations, analogs of ACV were synthesized in which the δ-(L-α-aminoadipyl) moiety was replaced by: β-(L-aspartyl) (**6**), γ-(L-glutamyl) (**7**), δ-(D-α-aminoadipyl) (**8**), adipyl (**9**), *N*-acetyl-δ-(L-α-aminoadipyl) (**10**), and glycyl-δ-(L-α-aminoadipyl) (**11**). Each of these peptides was then subjected to the action of the cyclizing enzyme of *S. clavuligerus*. To facilitate the identication of possible cyclization products, glycyl-isopenicillin N (**12**), *N*-acetylisopenicillin N (**13**), and carboxybutylpenicillin (**14**) were also synthesized. All of the peptides were supplied for enzymatic experiments in the disulfide form. Reduction to the monomeric peptides was performed immediately prior to assays, using dithiothreitol (3).

During the course of the work, two improvements were made in the published synthesis of ACV (23): a technically simpler preparation of *S*-trityl-L-cysteinyl-D-valine benzhydryl ester (**15**) was developed, based on preferential removal of a *tert*-butoxycarbonyl protecting group in the presence of a benzhydryl ester; and a new synthesis of L-α-aminoadipic acid from L-lysine was found. Difficulties encountered in the conversion of α-aminoadipic acid to *N*-acetyl-α-aminoadipic acid led to the development of a new reagent for *N*-acetylation under mild conditions in aqueous media. Details of all of these syntheses are provided.

Experimental

Materials

S-Trityl-L-cysteinyl-D-valine benzhydryl ester (15)

N-tert-Butoxycarbonyl-*S*-trityl-L-cysteinyl-D-valine benzhydryl ester (23) (2.00 g, 2.74 mmol) was dissolved in ethyl acetate (2.0 mL) to give a mobile yellow oil. To this was added 98% formic acid (200 mL) and the resultant solution was stirred vigorously at room temperature. After 15 min the reaction mixture was frozen using liquid nitrogen and then freeze-dried. The residual oil was azeotroped twice with methylene chloride, under reduced pressure, to leave a white foam. The crude formate salt was dissolved in ethyl acetate (100 mL) and washed with saturated sodium bicarbonate (2 × 80 mL, with brine added to minimize the formation of an emulsion), followed by saturated brine (80 mL). The solvent was dried over sodium sulfate and evaporated to leave a syrup which, after azeotroping with methylene chloride and drying under high vacuum, afforded a white foam, 1.49 g (87.5%).

A portion (724 mg) of the product was dissolved in chloroform (1.5 mL), diluted with hexane to the cloud point, and seeded to give white microprisms (517 mg), mp 155–156°C, $[\alpha]_D$ +34.7° (*c* 1.0, CH_2Cl_2); ir (KBr): 3355 (m), 3290 (w), 3160 (m, NH, NH_2), 1726, 1662 (s, ester, amide carbonyl) cm^{-1}; ^{1}Hmr (200 MHz, $CDCl_3$) δ:

0.74 (3H, d, J = 7 Hz, $CHCH_3$), 0.84 (3H, d, J = 7 Hz, $CHCH_3$), 1.46 (2H, br, NH_2), 2.17 (1H, m, collapses to quintet, J = 7 Hz, on irradiation at 4.55 ppm; valine C3 methine), 2.47 (1H, q, J = 9, 12 Hz) and 2.75 (1H, q, J = 4, 12 Hz, A and B quartets for cysteine methylene which collapse to two doublets, J = 12 Hz, on irradiation at 2.90 ppm), 2.90 (1H, q, J = 4, 9 Hz, cysteine methine), 4.55 (1H, q, J = 5, 9 Hz, collapses to doublet, J = 9 Hz, on irradiation at 2.17 ppm, valine C2 methine), 6.87 (1H, s, benzhydryl methine), 7.16–7.53 (~26H, aromatic and amide NH). *Anal.* calcd. for $C_{40}H_{40}N_2O_3S$: C 76.40, H 6.41, N 4.45; found: C 75.98, H 6.48, N 4.30.

Bis-β-(L-aspartyl)-L-cysteinyl-D-valine (6)

This compound was synthesized from **15** and N-BOC-L-aspartic acid-α-benzhydryl ester as described for ACV (23), except that the final deprotection was accomplished with formic acid (16 h). Thin-layer chromatography (tlc): R_f 0.12 (butanone – acetic acid – water, 4:1:1); ^{1}Hmr (D_2O) δ: 0.95 (12H, t, 6 Hz), 2.17 (2H, m), 2.99 (4H, m), 3.05 (2H, q, 8, 14 Hz), 3.18 (2H, q, 6.5, 14 Hz), 4.05 (2H, q, 6.5, 8 Hz), 4.20 (2H, d, 6.4 Hz), 4.8 (2H, m).

Bis-γ-(L-glutamyl)-L-cysteinyl-D-valine (7)

This compound was synthesized from **15** and N-BOC-L-glutamic acid-α-benzhydryl ester as described for **6**; tlc: R_f 0.12 (butanone – acetic acid – water, 4:1:1); ^{1}Hmr (D_2O) δ: 2.17 (4H, m), 2.51 (4H, m), 3.00 (2H, q, 8, 14 Hz), 3.18 (2H, q, 6, 14 Hz), 3.77 (2H, t), 4.17 (2H, d), 4.78 (2H, m).

Bis-δ-(D-α-aminoadipyl)-L-cysteinyl-D-valine (8)

This compound has $[\alpha]_D^{20}$ −35.8 (*c* 0.8, 2 *N* HCl); tlc: 0.18 (butanone – acetic acid – water, 4:1:1); ^{1}Hmr (D_2O) δ: 0.92 (12H, t, 7 Hz), 1.73 (4H, m), 1.88 (4H, m), 2.18 (2H, m), 3.00 (2H, q, 8.2, 15 Hz), 3.20 (2H, q, 6.5, 15 Hz), 3.78 (2H, br t), 4.21 (2H, d, 6 Hz), 4.81 (2H, m).

Bis-adipyl-L-cysteinyl-D-valine (9)

Adipic acid (730 mg, 5 mmol) was dissolved in a mixture of methanol (5 mL) and methylene chloride (30 mL), and a solution of diphenyldiazomethane (970 mg, 5 mmol) was added in portions during 8.5 h. When the addition was complete, the solvent was removed and the residue was triturated with ethyl acetate (15 mL) and filtered to give 220 mg of unreacted adipic acid. The filtrate was evaporated, ether (2 mL) was added, and this solution was treated with dicyclohexylamine (519 mg, 3.49 mmol). Hexane was then added to the cloud point and the mixture was chilled overnight. The crystals were then collected (1.1 g, mp 101–104°C) and recrystallized from ethyl acetate – ether (0.92 g, mp 103.5–105°C). The salt (420 mg) was partitioned between ethyl acetate (15 mL) and 10% potassium bisulfate (5 mL), and the organic layer was washed successively with 10% potassium bisulfate (2 × 5 mL) and brine (5 mL), dried, and evaporated. The residue crystallized from hexane (279 mg). Recrystallization from methylene chloride – hexane afforded 197 mg (mp 84.5–85.5°C) of the monobenzhydryl ester of adipic acid. Coupling of this ester with **15**, and further treatment as described for **6** led to **9**, R_f 0.66 (95% ethanol – acetic acid, 20:1).

Conversion of pipecolic acid to α-aminoadipic acid

Thionyl chloride (50.3 mL) was added dropwise, with cooling at 0°C, to a solution of pipecolic acid (50 g, 0.387 mol) in absolute methanol (400 mL). The reaction mixture was allowed to warm to room temperature, stirred for 12 h, and the solvent was then removed under reduced pressure. The crystalline residue was dissolved in dry methylene chloride (900 mL), the solution was cooled to 0°C, stirred, and dry triethylamine (110 mL, 0.785 mol) was added during 15 min followed, dropwise, by a solution of acetyl chloride (28 mL, 0.39 mol) in methylene chloride (100 mL). This mixture was stirred for 12 h, allowed to warm to room temperature, and water (750 mL) was added. The aqueous phase was extracted with methylene chloride (2 × 250 mL), and the combined organic phases were dried and evaporated. The residual oil was distilled (bp 100–110°C/2 Torr; 1 Torr = 133.3 Pa).

A mixture of methyl *N*-acetylpipecolate (46.4 g, 0.25 mol), ruthenium trichloride trihydrate (5 g), sodium periodate (1.073 L of 10% solution), and chloroform (1 L) was stirred vigorously. After 4 h the mixture turned black and was filtered through glass wool. The organic phase was separated, dried, and evaporated. The residue (47.1 g, 0.24 mol) was refluxed for 10 h in 8 *N* hydrochloric acid (1.41 L) and the resulting solution was then cooled to room temperature and concentrated under reduced pressure to a volume of 150 mL. The pH was adjusted to 2.9 with 10 *N* sodium hydroxide and the mixture was stored at 0°C overnight. Subsequent filtration afforded α-aminoadipic acid (24 g), identified by ir, nmr, and tlc.

Conversion of L-α-amino-ω-hydroxycaproic acid (24) to N-BOC-L-α-aminoadipic acid benzhydryl ester

To the amino acid alcohol **18** (92.5 mg, 0.63 mmol) in 50% acetone–water (1 mL) containing triethylamine (95.4 mg, 0.94 mmol) was added BOC-ON (155 mg, 0.63 mmol). The resulting yellow solution was stirred for 2 days and the solvent was then removed. The semi-solid residue was dissolved in water (40 mL) and this solution was extracted with ether (3 × 10 mL). These ether extracts were discarded. The aqueous phase was acidified with 10% potassium bisulfate and extracted with ethyl acetate (3 × 30 mL). The organic phase was dried and evaporated to afford the N-BOC derivative (135 mg, 87%) as a colourless oil. This oil (94.5 mg, 0.38 mmol) was dissolved in methylene chloride (2.5 mL) and treated with diphenyldiazomethane (74 mg, 0.38 mmol). After 5 min, the solvent was evaporated, and the residue was chromatographed (methylene chloride – ethyl acetate 5:1) to give the N-BOC benzhydryl ester (157 mg, 100%). A suspension of platinum oxide (100 mg) in water (1 mL) was stirred under a hydrogen atmosphere for 30 min, purged with nitrogen for 30 min, and then placed under an oxygen atmosphere. Sodium bicarbonate (32 mg, 0.38 mmol) was added, followed by the N-BOC benzhydryl ester (157 mg) in 66% acetone (5 mL). Stirring was continued under oxygen for 16 h and the mixture was then centrifuged and the supernatant acidified with 2% potassium bisulfate. Extraction with methylene chloride, followed by drying and evaporation of the organic extract, yielded N-BOC-L-α-aminoadipic acid benzhydryl ester (156 mg, 96%) as a semi-solid. Dicyclohexylamine salt: mp 156–158°C (lit. (23) mp 156.5–157.5°C); $[\alpha]_D^{25}$ −21.6° (*c* 2, methanol) (lit. (23) $[\alpha]_D$ −20.5 (*c* 2, methanol)).

2-Acetoximino-2-phenylacetonitrile

To 2-oximino-2-phenylacetonitrile (25) (3.1 g, 0.021 mol) in pyridine–benzene (10 mL of a 1:2 mixture) was added acetyl chloride (1.5 mL, 2.08 g, 0.026 mol). The reaction mixture was stirred at 0°C for 3 h, and the solvent was then removed under reduced pressure. The residue was dissolved in a mixture of water and ether and the organic layer was separated, dried, and evaporated. The crystalline residue was recrystallized from ether–hexane to give 3.1 g, mp 70–71°C; ^{1}Hmr ($CDCl_3$) δ: 2.28 (3H, s), 7.35–7.72 (5H, m). *Anal.* calcd. for $C_{10}H_8N_2O_2$: C 63.82, H 4.38, N 14.89; found: C 63.95, H 4.44, N 14.63.

Conversion of L-α-amino-ω-hydroxycaproic acid to N-acetyl-L-α-aminoadipic acid benzhydryl ester (21)

The amino acid alcohol **18** (73.5 mg, 0.5 mmol) was dissolved in 50% acetone (1.5 mL), and triethylamine (1.5 equiv., 100 μL) and 2-acetoximino-2-phenylacetonitrile (113 mg, 0.77 mmol) were added. The resulting solution was stirred at room temperature for 4 h, and the solvent was then removed under reduced pressure. The residue was dissolved in dry methanol (3 mL) and a solution of diphenyldiazomethane (100 mg, 0.52 mmol) in methylene chloride (0.5 mL) was added. The mixture was stirred for 30 min, concentrated, and the residue was chromatographed on silica gel. Elution with methylene chloride – methanol (10:1) gave 110 mg (62%) of **25**; $[\alpha]_D^{24}$ − 9.5° (*c* 2, chloroform); ir (KBr): 3270, 1730, 1650 cm^{-1}; ^{1}Hmr ($CDCl_3$) δ: 1.02–1.95 (6H, m, $CH_2CH_2CH_2$), 1.96 (3H, s, $COCH_3$), 3.51 (2H, t, 6 Hz, CH_2), 4.75 (1H, sextet, 5.5, 7.5 Hz, CH), 6.1 (1H, d, 7.5 Hz, NH), 6.9 (1H, s, CH), 7.33 (10H, s).

Platinum oxide (1 g) was suspended in 50% aqueous acetone (10 mL), containing sodium bicarbonate (168 mg, 2 mmol), and the suspension was stirred under hydrogen for 30 min, purged with nitro-

gen, and then placed under an oxygen atmosphere. The protected alcohol **25** (510 mg, 1.44 mmol) was added, and vigorous stirring was continued, under oxygen, for 12 h. The mixture was then centrifuged, the residue washed with water (2 × 2 mL), and the combined supernatant was evaporated under reduced pressure. The residue was dissolved in water, the pH was adjusted to 3.0, and the resulting precipitate was extracted with ethyl acetate. This extract was washed successively with 10% potassium bisulfate (10 mL), water (10 mL), and brine (10 mL), then dried and evaporated to yield **21** (340 mg, 64%) as a foam; $[\alpha]_D^{25}$ −7.8° (*c* 2, chloroform); ir (KBr): 1720, 1705, 1660 cm^{-1}; ^{1}Hmr ($CDCl_3$) δ: 1.35−1.95 (4H, m), 1.97 (3H, s), 2.27 (2H, m), 4.75 (1H, sextet, 5.5, 7.5 Hz), 6.29 (1H, d, 7.5 Hz), 6.88 (1H, s), 7.33 (10H, s).

N-Acetylation of L-α-aminoadipic acid-α-benzhydryl ester

The ester **22** (60 mg, 0.18 mmol) was suspended in 50% acetone (1.5 mL), and 2-acetoximino-2-phenylacetonitrile (36 mg, 0.19 mmol) and triethylamine (1.5 equiv.) were added. The suspension dissolved and the resulting solution was stirred for 4 h and then evaporated. Chromatography on silica gel (methylene chloride − methanol, 4:1) afforded 60 mg (90%) of **21**.

*Bis-N-acetyl-δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (**10**)*

This compound was synthesized from **15** and *N*-acetyl-L-α-aminoadipic acid-α-benzyhydryl ester; tlc: R_f 0.56 (butanone − acetic acid − water, 4:1:1); ^{1}Hmr (DMSO-d_6) δ: 0.96 (6H, d, 7.5 Hz), 0.98 (6H, d, 7.5 Hz), 1.76 (8H, m), 2.00 (6H, s), 2.22 (2H, m), 2.34 (4H, br t), 2.97 (2H, q, 9, 14 Hz), 3.20 (2H, q, 6, 14 Hz), 4.33 (2H, m), 4.38 (2H, m).

*Bis-glycyl-δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (**11**)*

This compound was synthesized from N-BOC-glycyl-L-α-aminoadipic acid-α-benzhydryl ester (26) and **15**: tlc: R_f 0.1 (butanone − acetic acid − water, 4:1:1), ^{1}Hmr (D_2O) δ: 0.89 (6H, d, 7 Hz), 0.94 (6H, d, 7 Hz), 1.70−2.30 (10H, m), 2.39 (4H, m), 2.98 (2H, q, 8.6, 14 Hz), 3.21 (2H, q, 5.5, 14 Hz), 3.87 (4H, s), 4.16 (2H, d, 6 Hz), 4.28 (2H, m), 4.76 (2H, q, 5.5, 8.6 Hz). *Anal.* calcd. for $C_{34}H_{58}N_8S_2O_{18}\cdot H_2O$: C 43.03, H 6.37, N 11.81; found: C 43.00, H 6.84, N 12.30.

*Glycylisopenicillin N (**12**)*

A solution of L-α-aminoadipic acid-α-benzhydryl ester (321 mg, 0.98 mmol) and dicyclohexylamine (0.4 mL) in methanol (16 mL) and dimethoxyethane (5 mL) was treated with *N*-carbobenzoxyglycine succinimidyl ester (306 mg, 1.0 mmol) (27), and then stirred at room temperature for 72 h. The solvent was removed, and the residue was redissolved in methylene chloride (25 mL), washed with 10% potassium bisulfate (3 × 30 mL) and brine (20 mL), dried, and evaporated. The resulting syrup (565 mg) was redissolved in methylene chloride (10 mL), and the solution was filtered and reevaporated to give a yellow foam (504 mg). A portion of this carbobenzoxyglycyl-L-α-aminoadipic acid-α-benzhydryl ester was purified by rapid chromatography; ^{1}Hmr ($CDCl_3$) δ: 1.58 (4H, m), 2.19 (2H, m), 3.87 (2H, m), 4.71 (1H, m), 5.08 (2H, s), 5.89 (1H, m), 6.86 (1H, s), 7.30 (16H, m), 9.45 (1H, br s). This compound was then converted to glycyl-isopenicillin N, as described for the syntheses of isopenicillin N and penicillin N (4); ir (KBr): 1768 cm^{-1}; ^{1}Hmr (D_2O) δ: 1.52 (3H, s), 1.65 (3H, s), 1.76 (4H, m), 2.40 (2H, m), 3.91 (2H, s), 4.26 (1H, s), 5.49 (1H, d, 4 Hz), 5.57 (1H, d, 4 Hz).

*N-Acetylisopenicillin N (**13**)*

This compound was synthesized from *N*-acetyl-L-α-aminoadipic acid-α-benzhydryl ester and the benzhydryl ester of 6-aminopenicillanic acid (4), as described for **12**, ir (KBr): 1767 cm^{-1}; ^{1}Hmr (D_2O) δ: 1.49 (3H, s), 1.62 (3H, s), 2.03 (3H, s), 1.65−2.45 (6H, m), 4.24 (1H, s), 5.47 (1H, d, 4.0 Hz), 5.57 (1H, d, 4.0 Hz).

*Carboxybutylpenicillin (**14**)*

This compound was synthesized from the monobenzhydryl ester of adipic acid and the benzhydryl ester of 6-aminopenicillanic acid, as described for **12**; ir (KBr): 1765 cm^{-1}; ^{1}Hmr (D_2O) δ: 1.49 (3H, s), overlapping with 1.56 (4H, br m), overlapping with 1.63 (3H, s), 2.17 (2H, m), 2.37 (2H, m), 4.25 (1H, s), 5.66 (1H, d, 3.6 Hz), 5.75 (1H, d, 3.6 Hz).

Organism and cultural conditions

Streptomyces clavuligerus NRRL 3585 was maintained and cultivated as previously described (3). Cells from 40-h cultures were harvested by filtration, washed, and resuspended to 1/10 of the original culture volume (approximately 1.8 mg dry weight/mL) in 0.05 *M* Tris-HCl buffer, pH 7.0, containing 0.1 m*M* dithiothreitol (TD buffer) for preparation of cell-free extracts.

Enzyme preparations

Washed cell suspensions were broken, in 25-mL aliquots, by sonic oscillation for 2 × 15 s at setting 5 with a Sonifier Cell Disruptor 350, Branson Sonic Power, using a standard flat-tipped probe (1.9 cm diameter). Broken cell suspensions were centrifuged 30 min at 25 000 × *g* to remove unbroken cells and cell envelope material. The resulting supernatant, termed cell-free extract, was partially purified by ammonium sulfate precipitation. Solid ammonium sulfate was gradually added to cell-free extract (typically 100 mL) with gentle stirring at 4°C. Material which precipitated between 40% and 70% saturation was collected by centrifugation (15 min at 25,000 × *g*) and resuspended to 1/10 of the original culture volume in TD buffer. This salt-precipitated cell-free extract was concentrated tenfold by ultrafiltration (PM-10 filter, Amicon Corporation), returned to its original volume with TD buffer, and again concentrated tenfold to remove residual ammonium sulfate. The resulting crude enzyme concentrate contained 40−60 mg protein/mL.

Partially purified cyclizing enzyme was prepared by anion exchange chromatography of the crude enzyme concentrate using DEAE-trisacryl resin, as described in ref. 5. This partially purified cyclizing enzyme is functionally pure, since it contains none of the other enzyme activities associated with penicillin/cephalosporin biosynthesis.

Assays

Cyclization activity was measured in a 0.4-mL standard cyclization assay system containing: ascorbic acid 2.8 m*M*, $FeSO_4$ 45 μ*M*, Tris HCl buffer (pH 7.0) 50.0 m*M*, and enzyme 0.1 mL. One hundred micrograms of each peptide substrate was reacted with 25 μL of 66.7 m*M* dithiothreitol for 15 min at 21°C before addition to the standard assay system. The mixtures were incubated for 2 h at 20°C and the reactions were then terminated by the addition of 0.4 mL of methanol. Under these conditions this corresponds to >90% consumption of ACV. Antibiotic formation was monitored by biological assay of reaction mixtures and by hplc analysis.

Biological assays of reaction mixtures were conducted using the agar diffusion procedure with *Micrococcus luteus* ATCC 9341 as the indicator organism.

The protein contents of cell-free extract and salt precipitated cell-free extract were determined by the method of Bradford (28).

High pressure liquid chromatographic analysis of reaction mixtures

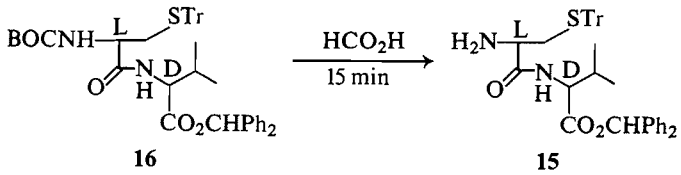
Methanol-inactivated reaction mixtures were centrifuged for 5 min at 12 000 × *g* before analysis. Equipment used for hplc analyses consisted of: an M6000 A pump, a WISP Model 710B automatic injector, and M-480 variable wavelength detector, an M420 data module, a Model 720 systems controller, and a C_{18} column (RadPak A in a Z module). All equipment was from Waters Scientific Ltd. The mobile phase consisted of methanol−KH_2PO_4 (0.05 *M* adjusted to pH 4.0 with H_3PO_4) in proportions which varied with the particular separation. The flow rate was 2 mL/min for 5 min and then 3 mL/min for the remaining analysis time. Detection of uv-absorbing materials was at 220 nm at a sensitivity of 0.02 absorbance units full scale.

Results

Syntheses of substrates

A. *Improved synthesis of* S-*trityl-L-cysteinyl-D-valine benzhydryl ester (**15**)*

In an earlier synthesis of ACV (23), **15** was prepared by *N*-detritylation of a *N*,*S*-ditritylated precursor. It has now been



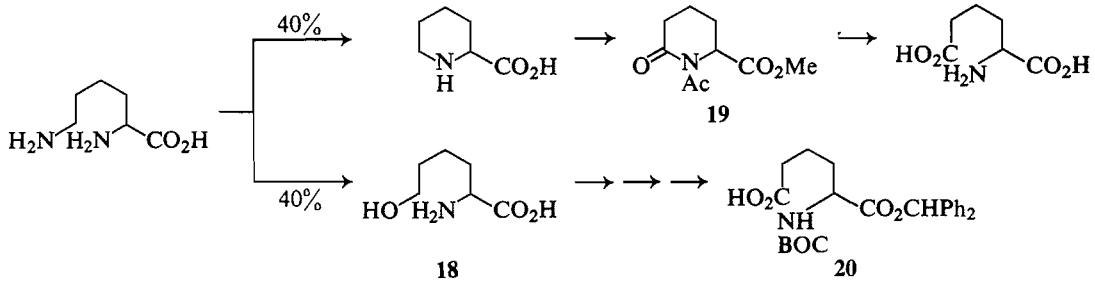
16 15

found that a 15-min treatment with a 98–100% formic acid leads to selective removal of the *tert*-butoxycarbonyl protecting group of **16** (23), and provides the intermediate **15** as a crystalline compound in over 80% yield. This is an improvement over the earlier sequence, because of the higher yield in the introduction of the nitrogen protecting group, and the greater ease of coupling of the cysteinyl and valinyl moieties when *N*-*tert*-butoxycarbonyl is employed in place of *N*-trityl.

B. *A new synthesis of* L-α-*aminoadipic acid*

Hungarian workers have recently observed deamination of amino acids, using sodium nitroprusside as the formal source of NO^+ (24). Applied to L-lysine, this reaction leads to L-pipecolic acid (**17**) (40%), and the amino acid alcohol **18** (40%). In the present work, oxidation of *N*-acetylpipecolic acid methyl ester with ruthenium tetroxide (29) led smoothly to **19**. Hydrolysis of this lactam provided α-aminoadipic acid.

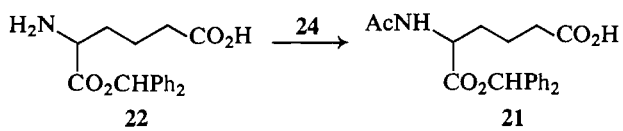
However, since α-aminoadipic acid is normally employed in our peptide syntheses in the form of the diprotected derivative **20**, the preparation of **20** from **17** requires six steps. The alcohol **18** is, therefore, a more useful intermediate since only three steps are then needed. In practice, these consisted of reaction with BOC-ON, reaction with diphenyldiazomethane, and oxidation with Pt/O_2. The yield of **20** from **18** was 83%.



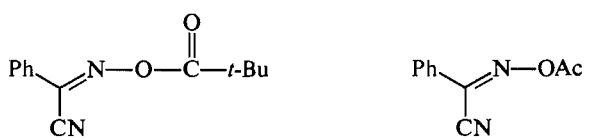
18 19 20

C. *A new reagent for* N-*acetylation in aqueous media*

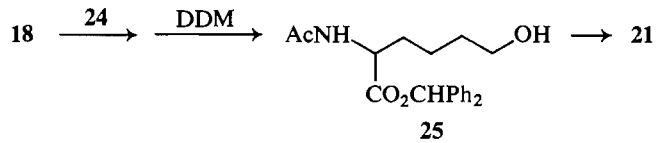
For the syntheses of the peptide **10** and the penicillin **13**, the *N*-acetylated α-aminoadipic acid derivative **21** was required. However, several attempts to acetylate **22** led to piperidone formation, and **21** could not be detected. Since BOC-ON (**23**) reacts smoothly with **22**, the analog **24** was synthesized from 2-oximino-2-phenylacetonitrile. This compound solved the problem. In aqueous acetone it acetylated **22** in 90% yield, and also *N*-acetylated the amino alcohol **18**.



22 21



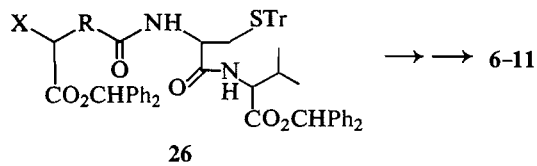
23 24



25

D. *Syntheses of peptides*

These followed the general strategy of ref. 23, with the modifications noted above. Condensation of **15** with the appropriately protected diacid or amino acid led to compounds of the general structure **26**. These were deprotected in two steps: detritylation with iodine in methanol; and removal of *tert*-butoxycarbonyl and benzhydryl protecting groups by overnight treatment with formic acid.

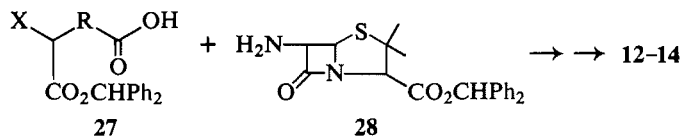


26

X = BOC-GLY, BOC-NH, AcNH, H

E. *Syntheses of penicillins*

These followed the strategy devised in ref. 4 for the syntheses of isopenicillin N and penicillin N. Condensation of the appropriately protected diacid or amino acid **27** with **28**, the benzhydryl ester of 6-APA (4) afforded the fully protected penicillins. These were deprotected by hydrogenolysis.



27 28

X = Z-GLY, AcNH, H

Cyclization of unnatural peptide substrates

A. *Biological assays*

In control reaction mixtures, none of the peptide substrates **1**, **6**–**11** exhibited antibacterial activity. Reaction mixtures containing enzyme, but no peptide substrate, also showed no activity.

Table 1 summarizes the results of experiments conducted under identical conditions for test reaction mixtures containing both substrate and enzyme. The L-aspartyl (**6**), L-glutamyl (**7**),

TABLE 1. Cyclization of aminoadipyl-modified analogs of ACV by cell-free extract from *S. clavuligerus*

Substrate	Zone of inhibition (mm)[a]
1 (ACV)	31.5 (30.5)[b]
6 (Asp-CV)	0
7 (Glu-CV)	0
8 (D-ACV)	0
9 (Ad-CV)	12.0 (12.0)[b]
10 (Ac-ACV)	28.0 (0)[b]
11 (GACV)	15.0 (0)[b]

[a]Twenty microlitre aliquots of methanol-inactivated reaction mixtures were bioassayed against *M. luteus* ATCC 9341 as the indicator organism.

[b]Data in parentheses refer to functionally purified cyclase. All other data refer to salt-precipitated cell-free extract.

TABLE 2. High pressure liquid chromatography retention times

Compound	Retention time (min) Method A[a]	Method B[b]
1	19.9	5.0
6	14.1	
7	17.5	
8	19.9	
9		14.1
10		6.3
11	21.7	
2	5.4	
5	5.4	
12	8.0	
13		2.9
14		8.3

[a,b]See text for description of these conditions.

and D-α-aminoadipyl (**8**) containing peptides are inactive substrates. However, antibacterial activity is observed when the adipyl (**9**), *N*-acetyl-L-α-aminoadipyl (**10**), and glycyl-L-α-aminoadipyl (**11**) containing peptides are employed as substrates.

B. *High pressure liquid chromatographic analyses*

The retention times of all monomeric peptide substrates and the putative cyclization products of the active substrates **1**, **9**−**11** were established prior to the analysis of test reaction mixtures. These are summarized in Table 2. Two sets of conditions were necessary because **9**, **10**, **13**, and **14** are retained by the stationary phase under the optimum conditions for elution of the other substrates and penicillins. For **1**, **6**, **7**, **8**, **11**, and **12**, the mobile phase consisted of 5% methanol − 95% KH_2PO_4 (0.05 *M* adjusted to pH 4.0 with H_3PO_4) (method A). For **9**, **10**, **13**, and **14**, the methanol content of the mobile phase was increased to 20% (method B).

Figure 1 shows hplc data obtained using method A, for a no-substrate control and for reaction mixtures containing ACV and **9**−**11**. In the case of the natural substrate ACV, the unreacted peptide is observed at 19.9 min, and there is a large peak at 5.4 min, corresponding to an unresolved mixture of isopenicillin N and penicillin N ((iso)penicillin N) (3−5). In the cases of the inactive substrates **6**−**8**, (data not shown in Fig. 1), hplc profiles of reaction mixtures are identical to the no-substrate control, with the addition of the peaks corresponding to unreacted peptide substrate. These peptides are, therefore, not undergoing transformation to biologically inactive products.

With GACV (**11**) as the substrate, unreacted peptide is observed at 21.7 min, and *(iso)penicillin N is observed at 5.4 min*. In addition, there is a minor peak, corresponding to ACV, at 19.9 min. The latter peak is not seen in control GACV reaction mixtures incubated without enzyme. Since no peak corresponding to glycyl-isopenicillin N is seen at 8.0 min, these observations represent a first indication (confirmed below) that GACV is converted in part to ACV under these conditions, and that the antibacterial activity reported in Table 1 refers to the cyclization of the latter compound.

A similar result is obtained with acetyl-ACV (**10**). The chromatogram shows a small ACV peak at 19.9 min, and a large (iso)penicillin N peak at 5.4 min. No peak is observed at 19.9 min in control reaction mixtures incubated without enzyme. However, the presence or absence of *N*-acetyl-ACV and *N*-acetylisopenicillin N in the reaction mixture cannot be established using method A, because of their retention by the stationary phase. For the same reason, the chromatogram, obtained using method A, of the reaction mixture containing adipyl-CV (**9**) is identical to that of the no-substrate control.

Figure 2 shows the hplc data obtained by method B for reaction mixtures containing **9** and **10**. With **10** as the substrate, all of the substrate is consumed, an ACV peak is seen at 5.0 min, but no penicillin peak corresponding to **13** can be found at 2.9 min. As will be seen below, the acetyl-ACV substrate has been converted completely to ACV *prior to* cyclization in the normal manner.

On the other hand, with **9** as the substrate, a considerable amount of the substrate remains unreacted, and is observed at 14.1 min. A new peak, corresponding to the penicillin **14**, is observed at 8.3 min. The low conversion of **9** that is observed in Fig. 2 is consistent with the low antibacterial activity noted in Table 1. Since zones of inhibition are related exponentially to the concentration of antibiotic, zones of 31.5 mm and 12 mm from ACV and from **9** would have suggested an antibiotic concentration about eighty times lower in the latter case, assuming the two antibiotics to have equal activity.

To check the conclusion that GACV (**11**) and acetyl-ACV (**10**) do not cyclize directly to (iso)penicillin N but, rather, undergo prior deacylation to ACV, cyclization assays were performed on these substrates in mixtures lacking iron and ascorbate. These are the essential cofactors for cyclization activity to be observed (3). Figure 3 shows the hplc data, using method A, obtained from such reactions. The no-substrate control differs in this case from that seen in Fig. 1, because the relative amounts of the oxidized and reduced forms of dithiothreitol (14.3 min and 9.3 min, respectively) are not the same in the absence of the cofactors. In particular, absence of ferrous ions permits the reduced form of dithiothreitol to persist for much longer times.

With **10** as the substrate for this experiment, the chromatogram shows a large ACV peak at 19.9 min, but no (iso)penicillin peak is observed at 5.4 min (and the reaction mixture shows no antibacterial activity). The chromatogram obtained using method B shows no residual **10**. On the other hand, GACV is somewhat more stable. The chromatogram obtained using method A shows unreacted GACV at 21.7 min,

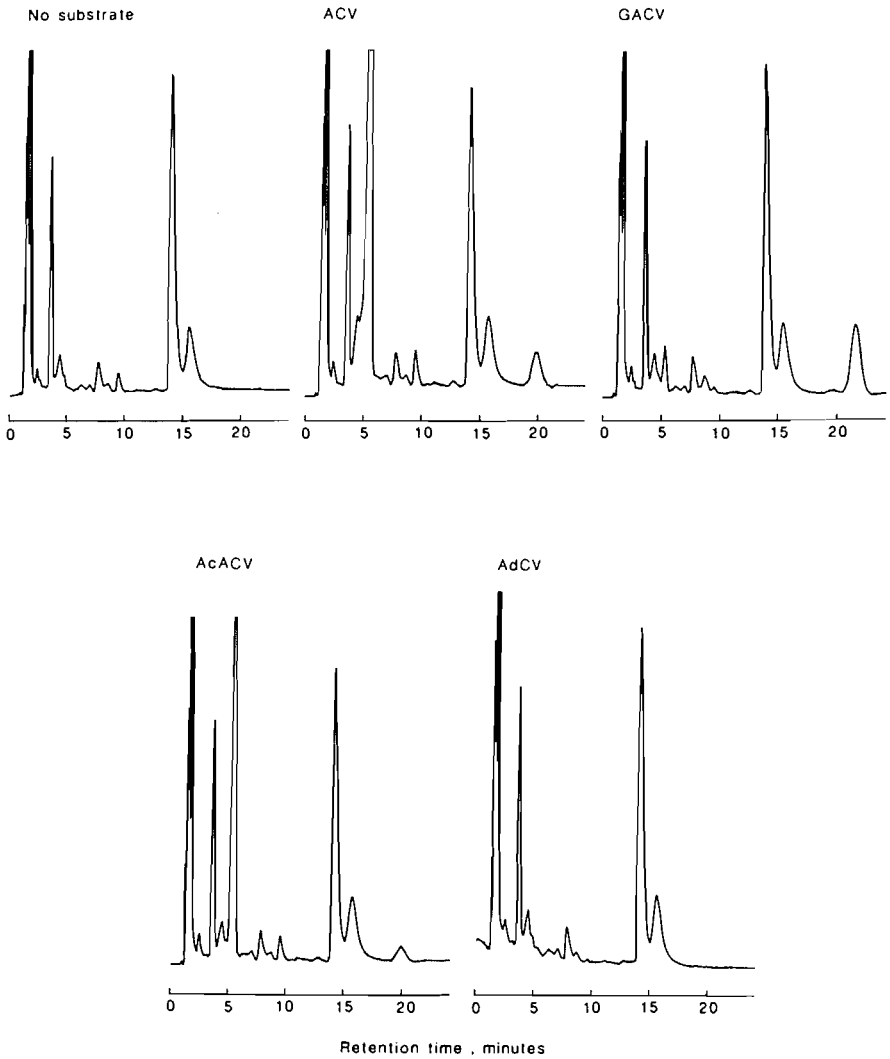


FIG. 1. Analysis of 2-h cyclization reaction mixtures by hplc with a mobile phase of 5% methanol − 95% KH_2PO_4 (0.05 *M* adjusted to pH 4.0 with H_3PO_4) (method A). Twenty microlitre aliquots of methanol-inactivated reaction mixtures were analyzed.

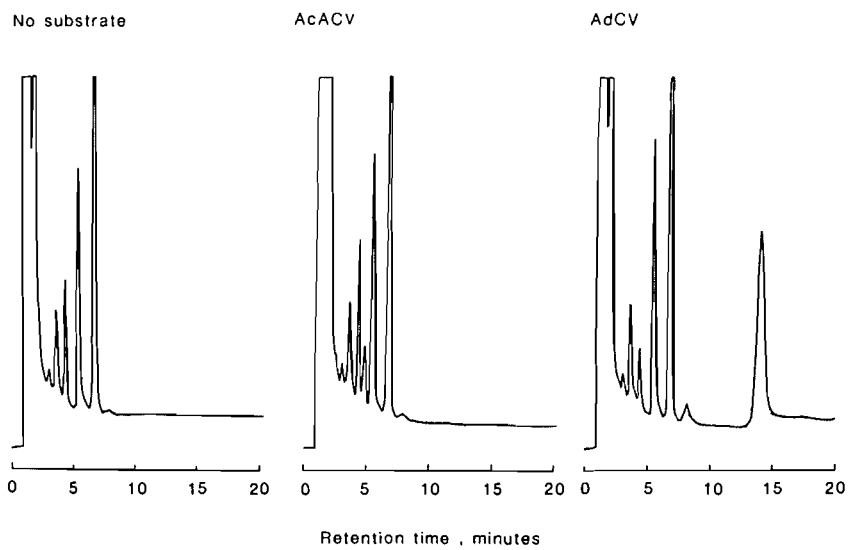


FIG. 2. Analysis of 2-h cyclization reaction mixtures by hplc with a mobile phase of 20% methanol − 80% KH_2PO_4 (0.05 *M* adjusted to pH 4.0 with H_3PO_4) (method B). Twenty microlitre aliquots of no-substrate control, AcACV and AdCV-containing cyclization reaction mixtures were analyzed after methanol inactivation.

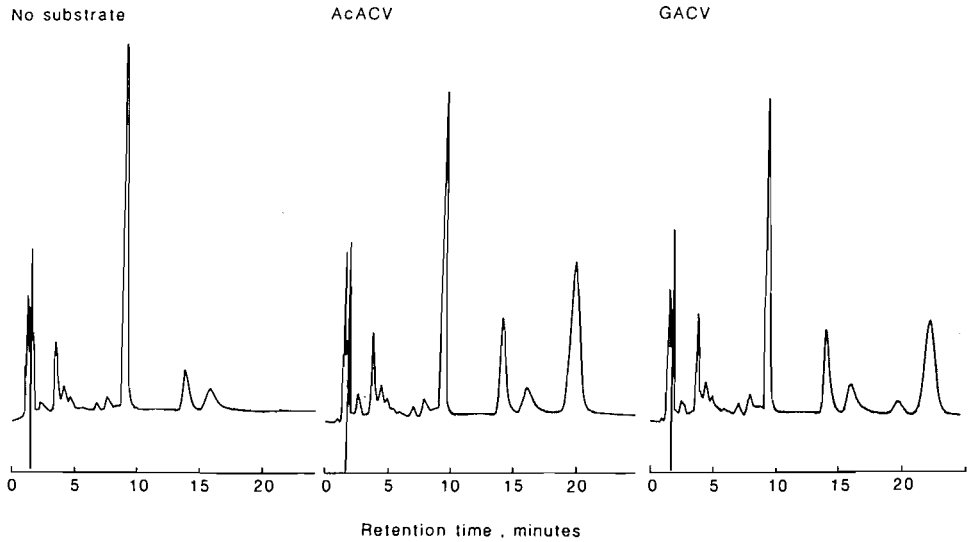


FIG. 3. The hplc analysis of cyclization reaction mixtures lacking iron and ascorbate using method A.

and ACV at 19.9 min. Again, no (iso)penicillin N is observed (and the reaction mixture shows no antibacterial activity).

It is clear that the activities observed from **10** and **11** in Table 1 reflect the extent to which these peptides were converted to ACV under the conditions of those experiments. Once formed, ACV resists further degradation, and there is no evidence that ACV is converted to CV or to free amino acids, either by *S. clavuligerus* cell-free extract or by non-specific proteases. Proteolytic activity, as measured by degradation of azocasein, was not detectable in cell-free extracts or semi-purified enzyme preparations. Similarly, addition of benzylsulfonyl fluoride to reaction mixtures at a final concentration of 1 m*M* had no effect on activity.

C. *Behaviour of functionally purified cyclizing enzyme*

The zones of inhibition shown in parentheses in Table 1 refer to cyclization experiments conducted with functionally purified enzyme. These data demonstrate that only ACV and adipyl-CV (**9**) are true substrates for the cyclizing enzyme, and confirm the conclusions reached in the preceding section.

Discussion

The present study has revealed that the cyclizing enzyme of *S. clavuligerus* has a high degree of specificity for the α-aminoadipyl moiety of ACV. Although this portion of the peptide is well removed from the sites of bond formation, and has no known role in the formation of the penicillin nucleus, modifications to this residue have a profound effect upon the activity of the peptide. A change in the configuration of the α-aminoadipyl residue from L to D or a decrease in the chain length by one or two carbons abolishes cyclizing activity. Although *N*-acylated derivatives of ACV exhibit some cyclization activity, the antibiotic produced in such cases is (iso)penicillin N, the natural product of ACV cyclization. That *N*-deacylation precedes cyclization with these substrates was demonstrated by the formation of ACV following preincubation of reaction mixtures under iron- and ascorbate-free conditions.

Of the analogs tested, only AdCV, which lacks the α-amino group of ACV, is an active substrate, but it is only 1−2% as active as ACV. All of these observations concerning the substrate specificity of the cyclizing enzyme of *S. clavuligerus* are very similar to those reported previously for the cyclizing enzyme of *C. acremonium* (6, 9).

Acknowledgements

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